Chapter 6

Incubation at 37°C prior to cryopreservation increases susceptibility of liver slices to ice crystal damage induced by rapid freezing*

*Based on:
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Abstract

Precision-cut liver slices are to some extent resistant to ice formation induced by rapid freezing. Susceptibility to rapid freezing damage has been shown to be (partly) dependent on intrinsic properties of cells. In the present study an attempt was made to decrease the susceptibility of rat liver slices to rapid freezing damage: to recover from low ATP levels, impaired ion regulation and cell swelling induced by their preparation, the slices were pre-incubated at 37°C under oxygen, prior to cryopreservation.

It was shown that unexpectedly recovery of cellular homeostasis prior to the cryopreservation procedure by the 37°C pre-incubation markedly decreased viability of rapidly frozen slices (in which ice was formed), but not of vitrified slices (in which no ice was formed), in a time- and temperature dependent manner. UW was found to protect slices from this ‘warm pre-incubation phenomenon’. Apparently, pre-incubation prior to freezing causes certain cellular alterations, that render slices more susceptible to ice crystals.
Slice pre-treatment increases susceptibility to ice crystal damage

Introduction

A considerable number of relatively successful methods for the cryopreservation of precision-cut liver slices have been developed in the past decade, with the goal to facilitate the use of these slices in \textit{in vitro} drug metabolism and pharmaco-toxicological research (Fisher et al., 1991; Wishnies et al., 1991; Fisher et al., 1993; de Kanter and Koster, 1995; Fisher et al., 1996; Ekins, 1996a; Ekins et al., 1996a; Glöckner et al., 1998; Day et al., 1998; Maas et al., 2000a; de Graaf et al., 2000b, Glöckner et al., 2001). Many of these cryopreservation methods are based on ‘rapid’ (200-1000°C/min) freezing and thawing of the slices after pre-incubation with moderately concentrated (12-30% v/v) DMSO solutions (de Kanter and Koster, 1995; Glöckner et al., 1998; Day et al., 1998; de Graaf et al., 2000b, Glöckner et al., 2001). An interesting feature of these methods is that reasonably high survival of the liver slices is obtained, even though the formation of inter- or even intracellular ice is most probably not prevented (de Graaf and Koster, 2001).

The resilience of cells to ice has been discussed by other authors. Survival of cells despite of exposure to ice crystals has been shown to be dependent on extrinsic (physical) factors, like the size and location of ice crystals (MacKenzie, 1970; Mazur, 1977; Hunt, 1984; Zieger et al., 1996), but also on intrinsic (biological) factors. The presence of intercellular connections is one of the intrinsic factors of cells reported to determine their survival after rapid freezing: isolated cells seem more prone to rapid freezing damage (and intracellular ice formation associated with it) than cells of the same type that are integrated within a tissue or monolayer (Zieger et al., 1996; Acker and McGann, 2000). Other intrinsic factors that determine the outcome of cryopreservation of liver slices involve the condition of the slices prior to cryopreservation, influenced by for example cold storage (Glöckner et al., 1996; de Graaf et al., 2000b) or the quality of liver donor material (Fisher et al., 2001).

In cryobiology research, focus is normally put on influencing \textit{extrinsic} factors that determine cryopreservation outcome, like prevention of intracellular ice formation or of cryoprotectant (CPA) toxicity. With the knowledge in mind that cells seem to be able to resist ice crystals under certain conditions, we decided to examine to which extent modification of \textit{intrinsic} factors, like tissue condition, could influence cryopreservation outcome. Our hypothesis was that improving the condition of slices prior to cryopreservation would positively influence survival of those slices after
thawing. In liver slice preparation, a period of warm ischemia during liver excision and low temperatures during preparation and storage of the slices is known to lead to an initially low cellular ATP content, disturbed cellular ion levels and cell swelling in freshly cut slices (Smith et al., 1986; Smith et al., 1987; Dogterom et al., 1993; Toutain et al., 1998; De Kanter et al., 1999; Maas et al., 2000a; Maas et al., 2000b and own unpublished observations). Upon subsequent incubation at physiological temperatures, cellular homeostasis is normally regained within 1-4 h. In the present study we attempted to improve slice condition by pre-incubating them at 37°C prior to cryopreservation. In this manner we allowed the slices to regain cellular homeostasis. Subsequently, slices were rapidly frozen after impregnation with 18% DMSO.

On the other hand, if improvement of slice condition indeed reduces its susceptibility to ice crystal damage, one would expect that this effect would be absent when slices are cryopreserved by vitrification (thus no ice crystals are formed within the slice). To confirm this, in the present study, some 37°C pre-incubated slices were vitrified after treatment with VS4 (a high molarity mixture of CPAs; (Fahy and Ali, 1997)) instead of rapidly frozen.

Viability of slices was assessed after 4 h of culturing after treatment by measuring intracellular potassium and/or ATP levels and by examining slice histomorphology, parameters that are considered to be very sensitive to cell damage (Maas et al., 2000b). We will show that, unexpectedly, pre-incubation prior to the cryopreservation procedure, markedly decreased viability of rapidly frozen slices, but not of vitrified slices, in a time- and temperature dependent manner. We will discuss what possible alterations within the liver slices could be inflicted during this warm pre-incubation period and how these alterations could render the slices more susceptible to ice crystal damage.
Materials and methods

Chemicals
Formamide, Krebs Henseleit (KH) buffer, HEPES buffer, insulin, gentamycin, adenine and glutathione were obtained from Sigma, Axel, The Netherlands; William’s Medium E/glutamax (WME), Phosphate buffered saline (PBS) and Fetal calf serum (FCS) were from Gibco BRL, Breda, The Netherlands; University of Wisconsin medium (UW) was from Lamepro b.v., Raamsdonkveer, The Netherlands; The Celsis Biomass assay kit was derived from Omnilabo, Breda, The Netherlands. The Coomassie Protein kit No. 23200, purchased from Pierce (Oud-Beyerland, The Netherlands). DMSO (>99.9% pure) and all other chemicals were from Baker, Deventer, The Netherlands.

Preparation of slices
For a more detailed description of the preparation and incubation of the slices, we refer to previous publications (de Graaf et al., 2000b; de Graaf and Koster, 2001). Shortly, the liver was excised from male rats (Wistar, obtained from Harlan CPB, Horst, The Netherlands) that had free access to food and water, after anaesthesia with 65% CO2, 35% O2. Depending on the experiment, sometimes the liver was perfused with UW prior to further treatment. Precision-cut liver slices were made from 8 mm tissue cores using a Krumdieck tissue slicer (Krumdieck et al., 1980) filled with ice-cold oxygenated KH buffer. In some experiments, other media were used for slicing. After preparation, slices were stored in ice-cold oxygenated WME with 10% FCS (or other media, dependent on the experiment) for approximately 45 min until further use.

To study the influence of regaining cellular homeostasis on cryopreservation outcome, slices were pre-incubated at 15, 25 or 37°C in oxygenated WME+supplements (for the description of the supplements, see the slice culturing section), for various time periods prior to the cryopreservation procedure. To study the influence of the pre-incubation medium, in some experiments UW was used instead of WME. In other experiments calcium chelators, allopurinol or oxygen radical scavengers were added to WME.
Cryopreservation and thawing

Slices were cryopreserved and thawed as previously described (de Graaf et al., 2000b). First, slices were impregnated with 18% DMSO in oxygenated WME on ice for 30 min under gentle shaking. Subsequently, they were brought over to cryovials together with 0.5 ml of the pre-incubation medium, and directly submerged into liquid nitrogen (cooling rate approximately 200°C/min). After storing the cryopreserved slices in liquid nitrogen, they were thawed by placing the cryovials in a 37°C waterbath until ice was no longer visible (warming rate approximately 200°C/min).

In some experiments, slices were vitrified instead of rapidly frozen. For this purpose, slices were impregnated stepwise with increasing concentrations of oxygenated VS4 solutions, like previously described (de Graaf and Koster, 2001). VS4 consists of DMSO, 1,2-propanediol and formamide (weight ratio 21.5: 15: 12.4 with a total concentration of 7.5M) mixed with saline, glucose, adenine and glutathione (for the exact composition see Fahy and Ali (1997)). Slices were vitrified by placing them between pads of aluminium foil followed by direct immersion in liquid nitrogen (cooling rate approximately 800°C/min (de Graaf and Koster, 2001)). Vitrified slices were thawed by placing the foil with the slices in ice-cold VS4 (warming rate approximately 800°C/min (de Graaf and Koster, 2001)). Subsequently, slices were washed stepwise in solutions with decreasing VS4 concentrations. To detect any adverse effects of VS4 on the slices, some slices were treated with VS4, not vitrified but directly incubated at 37°C for 4 h to test viability. In each experiment, one VS4 impregnated slice was taken and measured by Differential Scanning Calorimetry (DSC) as previously described (de Graaf and Koster, 2001) in order to determine whether the slices actually vitrified upon cooling and devitrification was prevented upon warming (thus the critical cooling and warming rates ($V_{cfc}$ and $V_{crw}$) were lower than the attained cooling and warming rates of 800°C/min).

Slice incubation and viability testing

Fresh and thawed slices were incubated in a 25-ml Erlenmeyer flask (1 slice/flask) in a shaking waterbath (110 times/min), under humid carbogen (95% O₂, 5%CO₂) in WME+glutamax, supplemented with FCS (5%), 0.1 µM insulin, 50 mg/l gentamycine and D-glucose (to a medium concentration of 25 mM). After culturing for 4 h at 37°C viability was determined.
The ATP content of liver and kidney slices was determined using a Celsis Biomass Assay kit, as follows: Slices were cut in two equal parts. One part was used for histomorphological examination (see below). The other part was immersed in 70% ethanol in HPLC water, containing 2 mM EDTA (pH 10.9). Then, slices were homogenized using a Branford sonifier (50% duty cycle, 5 s) for determination of ATP and potassium content. For determination of the ATP content, an aliquot of the homogenate was taken and mixed 1/1 with Nucleotide Releasing Medium from the assay kit. If necessary, the homogenate was subsequently diluted in 70% ethanol/2 mM EDTA to ensure the values were in the range of the counter. Thereafter, the solution was diluted 1/10 with 0.025M HEPES buffer to decrease the ethanol concentration. Subsequently, to 100 µl of the solution, 50 µl luciferin/luciferase solution from the assay kit was added and the amount of luminescence as a measure of ATP content was determined using a Lumac Biocounter M500 (Lumac, The Netherlands). ATP content was determined relative to the protein content of the slices. Slice protein and potassium were determined in the slice homogenate as described previously (de Graaf et al., 2000b), except that now an I-lab 600 automatical spectrophotometer (Instrumentation Laboratory, Lexington, USA) was used for the measurements.

For histomorphological examination, liver slices were fixed in 70% ethanol and further processed as described by de Graaf et al. (2000b). Slice viability was determined in the haematoxylin and eosin stained cross sections by estimating the percentage of viable cells. For determination of viability upon microscopical examination, nuclear shape and staining and cytoplasmatic staining were taken into account. It should be noted that also slice edges (1-2 cellayers at each edge) that are damaged by cutting the slices during slice preparation were included in viability scoring.

Hypothermic cell swelling can be assessed in slices by determination of the total tissue water (TTW) of the slice (Sundberg et al., 1991). TTW is defined as (the wet weight-slice dry weight)/dry weight (Little, 1964). The slice wet weight was measured after carefully removing medium attached to the slice with paper tissue. The slice was then dried overnight in a 60°C oven and hereafter the dry weight was measured.
Fig. 1 The influence of incubation at 37°C on cellular parameters prior to cryopreservation (closed symbols) and after cryopreservation (open symbols). (a) ATP content, (b) K⁺ content, (c) TTW and histomorphology. Parameters of slices were monitored after 37°C pre-incubation and CPA impregnation, so directly prior to freezing (closed symbols). Cellular parameters of cryopreserved slices were determined after 37°C pre-incubation, CPA impregnation, rapid freezing, thawing and 4 h of culturing (open symbols). Data-points represent the mean of 3 slices from one liver (+SD). For comparison: control slices (which were directly incubated for 4 h and not pre-incubated, treated with CPAs or cryopreserved) had a mean ATP content of 24.6 ± 1.3 nmol/mg protein, a K⁺ content of 0.68 ± 0.05 µmol/mg protein and 72.5 ± 3.5 % intact cells in the slice cross section.

Fig. 2 The influence of duration and temperature of pre-incubation prior to cryopreservation on viability of rapidly frozen slices after 4 h of culturing after thawing. (a) histomorphology (% of intact cells) and (b) ATP content. Bars represent the mean of 3-7 livers + SD, 3 slices were used per liver. Values are given as relative to those of control slices of the same experiment. Control slices were cultured for 4 h, but not impregnated with CPA or cryopreserved. Mean ATP content of fresh controls after 4 h of culturing: 19.05 ± 6.1 nmol/mg protein (7 livers), mean % of intact cells in slice cross section: 70.1 ± 6.4 (7 livers).


**Results and Discussion**

We hypothesized that allowing the slices to regain normal cellular homeostasis prior to freezing would improve cryopreservation results. Surprisingly, the opposite is true: 37°C incubation prior to cryopreservation actually allowed slices to regain cellular homeostasis, but at the same time deteriorated the viability of slices after cryopreservation by rapid freezing. Indeed the restoration of cellular homeostasis during pre-incubation at 37°C (Fig. 1, closed symbols) is shown by the cellular ATP and potassium levels gradually increasing and reaching levels after 2 h of incubation that are close to the maximum values reported by other authors to be reached after re-equilibration of slices at physiological temperatures (Miller *et al.*, 1993; Maas *et al.*, 2000b). As shown in Fig. 1c, in the wake of ATP and potassium levels, cellular water content is recovered to near physiological levels (2.08 gram water/gram dry weight as reported by Sundberg *et al.* (1991)). Slices that were cryopreserved after regaining homeostasis during the 37°C incubation, had a very low viability compared to slices that were not pre-incubated prior to freezing (compare open symbols after 120 min of pre-incubation with those at 0 min of pre-incubation). While viability of non-pre-incubated cryopreserved slices was reasonably high (approximately 40% (histomorphology) to 70% (potassium content), in slices that were pre-incubated for only 15 min at 37°C prior to freezing, no viable cells were detected after thawing and subsequent culturing and ATP and potassium levels were only 10-25% of fresh slice levels (compare Fig. 1, open symbols at t=0 with those at t=15 min). Remarkably, slices that were not pre-incubated prior to freezing, managed to increase their initially low ATP content from 7 nmol/mg protein at the moment of freezing (Fig 1a, closed symbols, t=0) to 12 nmol/mg protein after thawing and culturing (Fig. 1a, open symbols, t=0). Correspondingly, slice potassium content was increased from 0.12 µmol/mg protein directly prior to freezing (Fig 1b, closed symbols, t=0) to 0.5 µmol/mg protein after thawing and culturing (Fig. 1b, open symbols, t=0). On the opposite, slices that were pre-incubated at 37°C prior to freezing, lost most of the regained ATP and potassium after freezing, thawing and culturing.

Fig. 2 shows that the impact of the pre-incubation prior to freezing is not only time-, but also temperature-dependent. If a 15 min pre-incubation took place at 15°C, it even seemed to be beneficial to the slices. If the 15°C pre-incubation was persisted for more than 60 min, viability was decreased. A 25°C pre-incubation during 15 min decreased the percentage of intact
cells after cryopreservation, but not the ATP content. If pre-incubation was carried out at 37°C, both parameters showed a dramatic loss of viability of thawed slices.

Based on the foregoing, we may conclude that pre-incubation prior to freezing in a time- and temperature-dependent manner causes certain cellular alterations, that render slices more susceptible to cryopreservation by rapid freezing. The question rises whether the pre-incubation sets the slices to be more susceptible to ice formed within the slice or, alternatively, to other factors that are related to cryopreservation (such as CPA toxicity or chilling injury). To answer this question, we set up an experiment in which 37°C pre-incubated slices were either impregnated with 18% DMSO and rapidly frozen or impregnated with VS4 and vitrified by ultra-rapid freezing (cooling rate at least 800°C/min [de Graaf and Koster, 2001]; DSC measurements confirmed that the $V_{cr}$ and $V_{cru}$ of the VS4 treated slices were near or below the feasible cooling and warming rate of 800°C/min and negligible ice crystal formation occurred, data not shown). If the deleterious effect of warm pre-incubation prior to freezing indeed rendered slices more susceptible to ice crystal damage, this effect should only occur in rapidly frozen slices but not in vitrified slices. Fig. 3 shows the result of this experiment: Viability of slices that were cryopreserved directly after preparation and CPA impregnation by either rapid freezing or vitrification had a reasonably high viability after thawing. Viability of vitrified slices was not affected by 37°C pre-incubation, but viability of rapidly frozen slices was again markedly decreased. Hence, it can be concluded that warm pre-incubation renders slices more susceptible to ice crystal damage.

Possible explanations for the ‘warm pre-incubation phenomenon’
The question remains what possible alteration is provoked during 37°C pre-incubation that renders slices more susceptible to ice crystal damage. Remarkably, alterations caused by warm pre-incubation are lethal to slices after cryopreservation, but not to fresh slices. Since the effect of the pre-incubation is depending on temperature and is most pronounced if pre-incubation takes place at 37°C, obviously, activation of the cellular machinery is required for this phenomenon to occur.
Slice pre-treatment increases susceptibility to ice crystal damage

**Fig. 3** The effect of 37°C pre-incubation prior to cryopreservation on viability of rapidly frozen or vitrified liver slices after thawing and 4 h of culturing. (a) histomorphology (% of intact cells) and (b) ATP content. Bars represent the mean values ± SD of 4 (ATP) or 6 (histomorphology) livers, 3 slices were used per liver. Values are given as relative to those of control slices of the same experiment. Control slices were cultured for 4 h but not impregnated with CPA or cryopreserved. Mean ATP content of fresh controls: 20.1 ± 6.4 nmol/mg protein, mean % of intact cells was 71.9 ± 6.4. Pre-incubation with VS4 decreased ATP content of (37°C pre-incubated) fresh slices by approximately 25% and the % of intact cells by 5%.

From transplantation research it is known that if organs are re-oxygenized at physiological temperatures after a period of (cold) ischemia, ‘reperfusion’/reoxygenation injury may occur. Pre-incubation at 37°C (under 95% O₂) could have caused a similar effect in the slices in the present study. Reperfusion injury is often associated with oxygen radical production leading to lipid peroxidation. Production of free radicals is associated with ATP catabolism and calcium influx leading to activation of xanthine oxidase. Since ATP levels are low after slice preparation and the low potassium content indicates that regulation of ion concentration is impaired, free radicals are likely to be produced leading to lipid peroxidation during the 37°C pre-incubation. Peroxidation of lipids in cellular membranes decreases their fluidity (Garcia *et al*., 1997) and a decrease in membrane fluidity is associated with increased mortality of spermatozoa after cryopreservation (Giraud *et al*., 2000). So we may hypothesize that lipid peroxidation during warm pre-incubation may predispose the slices for additional cryopreservation damage.
Fig. 4 The effect of 37°C pre-incubation in either UW or WME prior to cryopreservation on viability of rapidly frozen liver slices after thawing and 4 h of culturing. (a) slice histomorphology (% of intact cells) and (b) ATP content. For this experiment, livers were perfused in situ with UW and slices were cut and stored in UW as well. Bars represent the mean values + SD of 2 livers, 3 slices were used per liver. Values are given as relative to those of control slices of the same experiment. Control slices were cultured for 4 h but not impregnated with CPA or cryopreserved. Mean ATP content of fresh controls: 22 ± 4.5 nmol/mg protein, mean % of intact cells was 69.9 ± 2.6. There was no effect of 37°C pre-incubation in UW on fresh slices.

UW, an organ preservation solution, is known to prevent reperfusion injury. Critical components of this solution are allopurinol, that inhibits xanthine oxidase, and GSH, an oxygen radical scavenger. Furthermore, UW has an ‘intracellular’ ion composition thus preventing calcium influx during ischemia (Southard and Belzer, 1993). With this in mind we hypothesized that perfusion of the liver with UW, followed by slice preparation and storage in UW might prevent the warm pre-incubation phenomenon. Fig. 4 shows viability of rapidly frozen slices of livers that were perfused in situ with UW. Slices were cut and stored in UW and subsequently either impregnated with 18% DMSO and cryopreserved (0 min pre-incubation), or pre-incubated at 37°C for 15 or 60 min in either UW or WME and than impregnated and cryopreserved. Apparently, UW completely protects slices from the warm pre-incubation effect, but only if it is used for all phases of slice preparation and the warm pre-incubation itself. However, the use of WME without calcium or WME with added calcium chelators did not prevent the pre-incubation effect, neither did the addition of allopurinol to WME, or the oxygen radical scavengers GSH, vitamin E and C (data not shown). Since these modifications of WME should prevent lipid peroxidation during 37°C
pre-incubation but did not prevent the deleterious effect of warm pre-incubation on cryopreservation outcome, the proposed oxygen free radical theory to explain the pre-incubation effect seems not to hold true.

Concluding remarks
In agreement with other reports we found that influencing the intrinsic properties of a tissue prior to cryopreservation can determine cryopreservation outcome. In the present study it was shown that pre-incubation under oxygenated conditions at physiological temperatures prior to cryopreservation renders slices more susceptible to ice crystal damage. UW apparently protects slices from the deleterious effects of warm pre-incubation, but probably not by prevention of oxygen free radical formation. Which cellular alteration is provoked by 37°C pre-incubation is not yet clear. Further research to this phenomenon seems worth the effort, since it may provide further insight into the mechanism of survival of cells that are cryopreserved at so called ‘supra-optimal’ cooling rates and may support the optimization of rapid freezing protocols for slices and other tissues.

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